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**BEFORE THE BOARD OF PATENT APPEALS  
AND INTERFERENCES**

Paper No. 20040401

Application Number: 09/485,045  
Filing Date: May 12, 2000  
Appellant(s): LEE ET AL.

\_\_\_\_\_  
Lisa A. Haile  
For Appellant

**EXAMINER'S ANSWER**

This is in response to the appeal brief filed 22 January 2004.

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**(1) *Real Party in Interest***

A statement identifying the real party in interest is contained in the brief.

**(2) *Related Appeals and Interferences***

The brief contains a statement that there are no related appeals and interferences which will directly affect or be directly affected by or have a bearing on the decision in the pending appeal is contained in the brief.

**(3) *Status of Claims***

The statement of the status of the claims contained in the brief is correct.

**(4) *Status of Amendments After Final***

The appellant's statement of the status of amendments after final rejection contained in the brief is correct.

**(6) *Issues***

The appellant's statement of the issues in the brief is correct.

**(7) *Grouping of Claims***

Appellant's brief includes a statement that claims 2, 4-11, and 53-55 do not stand or fall together and provides reasons as set forth in 37 CFR 1.192(c)(7) and (c)(8).

**(8) *Claims Appealed***

The copy of the appealed claims contained in the Appendix to the brief is correct.

**(9) *Prior Art of Record***

Massagué, J. Ann. Rev. Biochem., 1998, vol. 67, pp. 753-791.

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**(10) Grounds of Rejection**

The following ground(s) of rejection are applicable to the appealed claims:

Claims 2, 4-11, and 53-55 are rejected under 35 U.S.C. 101. This rejection is set forth in prior Office Action of 19 May 2003 and restated below.

Claims 2, 4-11, and 53-55 are drawn to a polynucleotide encoding a GDF-16 protein, the complement of this polynucleotide, and host cells and vectors comprising these polynucleotides. The claimed invention is not supported by either a specific and substantial asserted utility or a well-established utility.

A specific and substantial utility is one that is particular to the subject matter claimed and that identifies a "real world" use for the claimed invention. See *Brenner v. Manson*, 148 U.S.P.Q. 689 (1966):

The basic quid pro quo contemplated by the Constitution and the Congress for granting a patent monopoly is the benefit derived by the public from an invention with substantial utility. . . . [u]nless and until a process is refined and developed to this point-where specific benefit exists in currently available form-there is insufficient justification for permitting an applicant to engross what may prove to be a broad field.

While Appellant lists a number of conditions for which the protein encoded by the GDF-16 polynucleotide might be used (p.5 and 6), the specification does not disclose any activity known to be associated with it. Merely listing a number of possibilities is not sufficient to identify or confirm a "real world" context of use; clearly further research would be required to ascertain the function of the encoded protein and the purposes for which it could be used. Similarly, no diseases or conditions with which the protein is associated are disclosed; thus, there is no "specific benefit in currently available form" to be derived from detection of GDF-16 or

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inhibition of its activity. Thus, further research is required to identify a disease for which it could be used, a disease in which its activity could be beneficially affected, or a disease for which its presence would be diagnostic. See *Brenner v. Manson*, noting that "a patent is not a hunting license. It is not a reward for the search, but compensation for its successful conclusion." A patent is therefore not a license to experiment.

The invention also lacks a well-established utility. A well-established utility is a specific, substantial, and credible utility that is well known, immediately apparent, or implied by the specification's disclosure of the properties of a material. The specification fails to assert any activity for the encoded protein other than those generally recognized to be attributes of TGF- $\beta$ . Identifying a polynucleotide as encoding a protein of this family does not endow the polynucleotide with a specific and substantial utility. Proteins of this family are known to function in many different physiological contexts: Massagué (*Ann. Rev. Biochem.*, 1998, vol. 67, pp. 753-791) teaches that "this family comprises a large number of ... factors, each capable of regulating a fascinating array of cellular processes..." (p. 745). Even those family members identified as GDFs have functions as varied as the promotion of chondrogenesis and the inhibition of muscle growth (p. 755). There is therefore no well-established utility for members of this family; they are involved in many different processes and utility is specific to the individual protein.

Claims 2, 4-11, and 53-55 are also rejected under 35 U.S.C. 112, first paragraph. Specifically, since the claimed invention is not supported by either a specific or substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention.

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**(11) Response to Argument**

Appellant argues that a number of utilities were asserted for GDF-16 based on its identification as a TGF- $\beta$  family member. These include growth and differentiation activity and use as a marker for a cell proliferative disorder. Appellant argues that the specification discloses that GDF-16 can be used to detect and diagnose a cell proliferative disorder by detecting an altered level of expression, and that it can be used to detect close family members. Appellant argues that these are well-established utilities. Appellant further argues that a number of papers available at the time described specific expression of TGF- $\beta$ 4 gene in cell proliferative disorders. Thus, Appellant concludes, GDF-16 polynucleotides could be used to detect TGF- $\beta$  family members such as TGF- $\beta$ 4 whose expression is correlated with cell proliferative disorders, and this is a well-established utility. Appellant argues that this utility would have been apparent to a skilled artisan, although it was not explicitly asserted in the specification. Appellant argues that the similarity of the GDF-16 polynucleotide to TGF- $\beta$ 4 is not fortuitous because it was identified using a TGF- $\beta$  family member probe. Appellant points to the utility guidelines as indicating that a protein differentially expressed in heart disease has utility, and asserts that, based on the identification of GDF-16 as a TGF- $\beta$  family member, the artisan would recognize that it could be used as a probe for closely related members such as TGF- $\beta$ 4.

Appellant argues that because GDF-16 is a TGF- $\beta$  family member it is likely that it can be used to detect a cell proliferative disorder and that it can be used to detect a close family member. Since these utilities are not applicable to all polynucleotides, Appellant argues, they are specific. Since they are related to important health issues, Appellant argues, they are substantial.

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Appellant argues that the credibility of the asserted utilities is supported by the published literature that indicates that the various TGF- $\beta$  family members have the asserted utilities.

For these reasons, Appellant further argues that, since GDF-16 has utility, one of skill would also be able to use it and thus the invention is enabled under 35 U.S.C. 112, first paragraph.

Appellant's arguments have been fully considered but have not been found to be persuasive. The specification fails to assert any specific and substantial utility for GDF-16. What is provided are general statements that it may have applications in "treating disease processes involving muscle" (p. 4, lines 26), in treating "immunologic disorders" (p. 5, line 8), in wound repair (p. 5, line 14), in regulation of fertility (p. 5, line 24), and in "various types of cancer" (p. 6, line 3). No actual activity of the GDF-16 protein itself is disclosed; the assertions are, as Appellant states, based on the identification of GDF-16 as a member of the TGF- $\beta$  family. There is no indication as to how GDF-16 might be involved in any of these conditions, what cells it affects, and whether it promotes or antagonizes cellular processes. Clearly, further research would be required to determine what the protein does and how it and the encoding polynucleotide could be used, or how GDF-16 proteins or polynucleotides could be used to diagnose any disease. Thus, no specific, substantial utility for GDF-16 itself is disclosed. That it is a member of the TGF- $\beta$  family does not provide it with such a specific and substantial utility. Massaguè lists 27 members of this family (table 1, p. 755). They are involved in gastrulation, neurogenesis, chondrogenesis, osteogenesis, induction of FSH, inhibition of FSH, monocyte chemotaxis, and immunosuppression. One family member inhibits muscle growth. Thus, identification of GDF-16 as a member of this family does not identify any particular function for

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it. It could be involved in many processes in many ways and the skilled artisan would not know what it did merely because it was a member of the TGF- $\beta$  family.

The specification provides general teachings that the polynucleotide could be used to identify related molecules. It does not disclose the relationship of GDF-16 to TGF- $\beta$ 4, nor does it identify any specific disease that GDF-16 could be used to diagnose. The prior art teaches TGF- $\beta$ 4 and its utility as a cancer marker. However, this utility is not specific to GDF-16 because it does not depend on any properties of GDF-16. The function of GDF-16 remains unknown and nothing is known about the expression of GDF-16 in any cancers. This utility thus depends solely on its similarity to another polynucleotide, not on any properties of GDF-16 itself, and neither the similarity nor this use was contemplated in the specification. Detection of TGF- $\beta$ 4 is not a well-established utility that would have been readily apparent from the identification of GDF-16 as a TGF- $\beta$  family member. According to the utility guidelines (Federal Register, vol. 66, no.4, January 5, 2001), an invention has a well-established utility if one of ordinary skill would immediately appreciate why the invention is useful “based on the characteristics of the invention (e.g. properties or applications of a product or process)”. The diversity of the family members, which have as little as 20% homology to each other (Massaguè, p. 755), is such that artisan would not have assumed it could be used to identify any other member and particularly not a specific member whose existence is not disclosed in the specification. Neither the instant specification nor the prior art cited by Appellant indicate that the identification of a polynucleotide as a member of the TGF- $\beta$  family would allow one to use it to identify TGF- $\beta$ 4 and diagnose cancer.



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Appellant argues that the similarity to TGF- $\beta$ 4 was not fortuitous because GDF-16 was identified using another family member. However, the method of identification would only allow the artisan to conclude that GDF-16 would be similar to the family member that was used to identify it, murine lefty. It would not point the artisan to any other particular member of the family. What would be required would be for the skilled artisan to compare sequences of GDF-16 with other family members, recognize the similarity to TGF- $\beta$ 4, and, from reading teachings specific to TGF- $\beta$ 4, conclude that GDF-16 could be used to detect TGF- $\beta$ 4 and thus diagnose cancer. This utility is therefore in no way “readily apparent”. Appellant’s general teachings that GDF-16 could be used to identify other molecules are merely an invitation to experiment to identify those molecules and to determine whether those molecules, once identified, are useful.

Appellant argues that the uses of GDF-16 to detect cell proliferative disorders and to detect related family members are specific because they do not apply to all polynucleotides, and substantial because they relate to human health. However, as stated above, the general assertions of usefulness in the detection of “cell proliferative disorders” and related molecules do not provide GDF-16 with a substantial utility. See *Brenner v. Manson*, 148 U.S.P.Q. 689 (1966), in which the court expressed the opinion that, while, all chemical compounds are “useful” to the chemical arts when this term is given its broadest interpretation, this broad interpretation was not the intended definition of “useful” as it appears in 35 U.S.C. §101, which requires that an invention must have either an immediately apparent or fully disclosed “real world” utility. There is no such “real world utility” associated with a general teachings that GDF-16 is somehow involved in cell proliferation and that it could be used to identify molecules it is related to. There is no “specific benefit in currently available form” associated with these teachings; they are, as

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stated above, merely an invitation to experiment to identify diseases that might be associated with GDF-16 and related molecules that might themselves be found to be useful.

Appellant argues that the credibility of the asserted utilities is supported by the published literature. The credibility of Appellant's asserted utilities is not challenged. However, they do not provide the invention with a specific and substantial utility for the reasons set forth above.

Since, for the reasons set forth above, Appellant's arguments have not been found to be persuasive and it is maintained that the claimed invention is not supported by either a specific or substantial asserted utility or a well established utility, it is similarly maintained that one skilled in the art would not know how to use the claimed invention.

For the above reasons, it is believed that the rejections should be sustained.

Respectfully submitted,

  
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## TGF- $\beta$ SIGNAL TRANSDUCTION

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KEY WORDS: TGF- $\beta$ , receptor serine/threonine kinases, SMADs, growth factors, development, cancer

### ABSTRACT

The transforming growth factor  $\beta$  (TGF- $\beta$ ) family of growth factors control the development and homeostasis of most tissues in metazoan organisms. Work over the past few years has led to the elucidation of a TGF- $\beta$  signal transduction network. This network involves receptor serine/threonine kinases at the cell surface and their substrates, the SMAD proteins, which move into the nucleus, where they activate target gene transcription in association with DNA-binding partners. Distinct repertoires of receptors, SMAD proteins, and DNA-binding partners seemingly underlie, in a cell-specific manner, the multifunctional nature of TGF- $\beta$  and related factors. Mutations in these pathways are the cause of various forms of human cancer and developmental disorders.

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The transforming growth factor  $\beta$  (TGF- $\beta$ ) family comprises a large number of structurally related polypeptide growth factors, each capable of regulating a fascinating array of cellular processes including cell proliferation, lineage determination, differentiation, motility, adhesion, and death. Expressed in complex temporal and tissue-specific patterns, TGF- $\beta$  and related factors play a prominent role in the development, homeostasis, and repair of virtually all tissues in organisms, from fruitfly to human. Collectively, these factors account for a substantial portion of the intercellular signals governing cell fate.

TGF- $\beta$  and related factors are multifunctional agonists whose effects depend on the state of responsiveness of the target cell as much as on the factors themselves. Given this multifunctional nature, it is not surprising, in retrospect, that the gradual discovery of these factors over the past 15 years has been made through very disparate lines of investigation. For example, the founding member of the family, TGF- $\beta$ 1, was identified as a regulator of mesenchymal growth and, separately, as an antimitogen in epithelial cells (see Table 1 for references). Activins were identified as endocrine regulators of pituitary function and, independently, as inducers of mesoderm in frogs. Bone morphogenetic proteins (BMPs) were identified as bone repair factors and, independently, as dorsalizing agents in *Drosophila*.

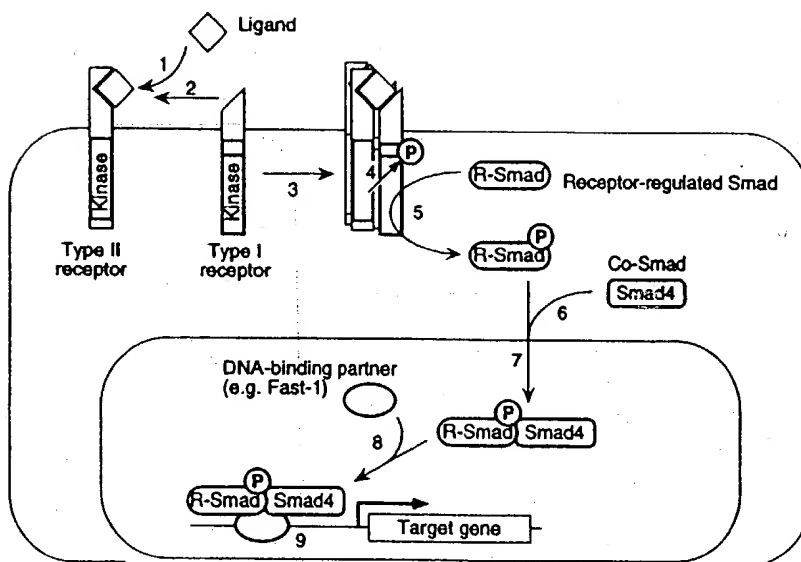
A listing of the current members of the TGF- $\beta$  family and their most representative activities is presented in Table 1 along with citations of articles that review in depth the discovery and biology of these factors. Based on sequence comparisons between the bioactive domains, the TGF- $\beta$  family can be ordered around a subfamily that includes mammalian BMP2 and BMP4 and their close homologue from *Drosophila*, Dpp. All other known family members progressively diverge from this group, starting with the BMP5 subfamily, followed by the GDF5

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Table 1 The transforming growth factor  $\beta$  (TGF- $\beta$ ) family and representative activities<sup>a</sup>

Names (Homologues)	%	Representative activities (References)
<b>BMP2 subfamily</b>		
BMP2 [Dpp <sup>D</sup> ]	100	Gastrulation, neurogenesis, chondrogenesis, interdigital
BMP4	92	apoptosis; in frog: mesoderm patterning; in fly: dorsalization, eyes, wings. (1-3)
<b>BMP5 subfamily</b>		
BMP5 [60 A <sup>D</sup> ]	61	Along with BMPs 2 and 4, this subfamily participates in the
BMP6/Vgr1	61	development of nearly all organs; many roles
BMP7/OP1	60	in neurogenesis. (1, 2)
BMP8/OP2	55	
<b>GDF5 subfamily</b>		
GDF5/CDMP1	57	Chondrogenesis in developing limbs. (1, 4)
GDF6/CDMP2	54	
GDF7	57	
<b>Vgl subfamily</b>		
GDF1 [Vgl <sup>X</sup> ]	42	Vgl: axial mesoderm induction in frog and fish. (4)
GDF3/Vgr2	53	
<b>BMP3 subfamily</b>		
BMP3/osteogenin	48	Osteogenic differentiation, endochondral bone formation,
GDF10	46	monocyte chemotaxis. (5)
<b>Intermediate members</b>		
Nodal [Xnr 1 to 3 <sup>X</sup> ]	42	Axial mesoderm induction, left-right asymmetry. (1, 6)
Dorsalin	40	Regulation of cell differentiation within the neural tube. (7)
GDF8	41	Inhibition of skeletal muscle growth. (8)
GDF9	34	
<b>Activin subfamily</b>		
Activin $\beta$ A	42	Pituitary follicle-stimulating hormone (FSH) production,
Activin $\beta$ B	42	erythroid cell differentiation; in frog, mesoderm
Activin $\beta$ C	37	induction. (3, 9, 10)
Activin $\beta$ E	40	
<b>TGF-<math>\beta</math> subfamily</b>		
TGF- $\beta$ 1	35	Cell cycle arrest in epithelial and hematopoietic cells, control of
TGF- $\beta$ 2	34	mesenchymal cell proliferation and differentiation, wound
TGF- $\beta$ 3	36	healing, extracellular matrix production, immunosuppression. (11-14)
<b>Distant members</b>		
MIS/AMH	27	Müllerian duct regression. (15, 16)
Inhibin $\alpha$	22	Inhibition of FSH production and other actions of activin. (9, 10)
GDNF	23	Dopaminergic neuron survival, kidney development. (17)

<sup>a</sup>All members listed have been identified in human and/or mouse. In brackets, important homologues from *Drosophila* (<sup>D</sup>) and *Xenopus* (<sup>X</sup>). %, percent of amino acid identity with human bone morphogenetic protein (BMP)2 over the mature polypeptide domain. GDF, growth and differentiation factor. CDMP, cartilage-derived morphogenetic protein. MIS/AMH, Müllerian inhibiting substance/anti-Müllerian hormone. GDNF, glial cell-derived neurotrophic factor.



**Figure 1** The transforming growth factor  $\beta$  (TGF- $\beta$ )/SMAD pathway. Binding of a TGF- $\beta$  family member to its type II receptor (1) in concert with a type I receptor (2) leads to formation of a receptor complex (3) and phosphorylation of the type I receptor (4). Thus activated, the type I receptor subsequently phosphorylates a receptor-regulated SMAD (R-Smad) (5), allowing this protein to associate with Smad4 (6) and move into the nucleus (7). In the nucleus, the SMAD complex associates with a DNA-binding partner, such as Fast-1 (8), and this complex binds to specific enhancers in target genes (9), activating transcription.

(growth and differentiation factor 5) subfamily, the Vg1 subfamily, the BMP3 subfamily, various intermediate members, the activin subfamily, the TGF- $\beta$  subfamily, and finally several distantly related members (Table 1) (1–17).

This review is devoted to a major accomplishment in this field over the past few years: the elucidation of a general mechanism by which TGF- $\beta$  and related factors activate receptors at the cell surface and transduce signals to target genes (Figure 1). Some of these genes encode immediate effectors of ultimate cellular responses, such as cell cycle regulators that mediate antiproliferative responses or extracellular matrix components that determine cell adhesion, positioning, and movement. TGF- $\beta$  and related factors regulate gene expression by bringing together two types of receptor serine/threonine protein kinases. One of these kinases phosphorylates the other, which in turn phosphorylates SMAD proteins. SMADs are a novel family of signal transducers that move into the nucleus and generate transcriptional complexes of specific DNA-binding ability. This review focuses on the structure and function of the TGF- $\beta$  receptor family and

the SMAD family, their mechanisms of activation and regulation, and their disruption in human disease.

## SIGNALING RECEPTORS

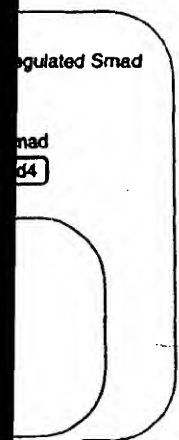
TGF- $\beta$  and related factors signal through a family of transmembrane protein serine/threonine kinases referred to as the TGF- $\beta$  receptor family. This family came to light with the cloning of an activin receptor (18), now referred to as ActR-II, with properties similar to those of TGF- $\beta$  receptors identified in ligand cross-linking studies (19) and genetically implicated in TGF- $\beta$  signal transduction (20). The cloning of ActR-II also revealed a striking similarity between this molecule and Daf-1, a previously identified orphan receptor from *Caenorhabditis elegans* (21). These findings provided the basis and impulse for the rapid identification of many other members of this receptor family.

Extensive evidence has accumulated to indicate that TGF- $\beta$  family members signal through receptor serine/threonine kinases. One exception is the glial cell-derived neurotrophic factor (GDNF), which signals through the receptor tyrosine kinase Ret (17). GDNF was included in the TGF- $\beta$  family because it has a set of cysteines that are characteristic of this family (22). However, GDNF is the most divergent family member and shows very little sequence similarity to other members (see Table 1). The next most divergent member, the Müllerian inhibiting substance (MIS; also known as anti-Müllerian hormone, AMH), signals through a TGF- $\beta$  receptor family member, AMHR (23). GDNF therefore is in a class of its own aligned with the structurally diverse group of factors that signal through receptor tyrosine kinases.

### Type I and II Receptor Families

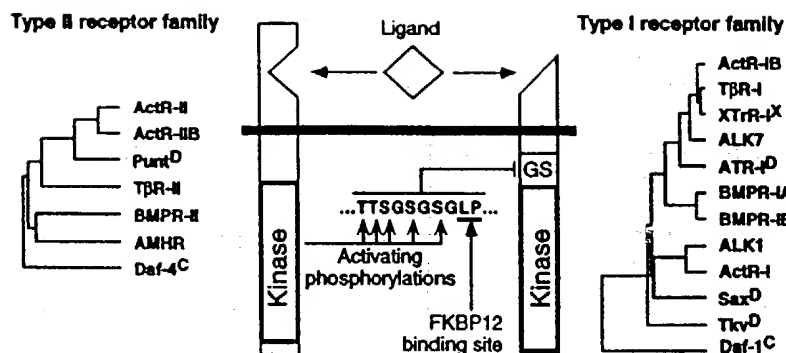
Based on their structural and functional properties, the TGF- $\beta$  receptor family is divided into two subfamilies: type I receptors and type II receptors (Figure 2). Type I receptors have a higher level of sequence similarity than type II receptors, particularly in the kinase domain. Vertebrate type I receptors form three groups whose members have similar kinase domains and signaling activities. In mammals, one group includes T $\beta$ R-I, ActR-IB, and ALK7, another includes BMPR-IA and -IB, and the third includes ALK1 and ALK2.

As a result of being simultaneously cloned by different groups, most type I receptors have received different names. One practice has been to use the neutral nomenclature ALK (activin receptor-like kinase) and to adopt a more descriptive name when the physiological ligand becomes known. Thus, the TGF- $\beta$  type I receptor originally known as ALK5 (24) is now called T $\beta$ R-I (25). ActR-IB (previously also known as ALK4) (26) is an activin type I receptor (27), and BMPR-IA and -IB (previously known as ALK3 and ALK6, respectively) are



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**Figure 2** Type I and II TGF- $\beta$  receptor families. In type I receptors, the protein kinase domain is preceded by the GS domain (GS). The characteristic GS sequence motif of T $\beta$ R-I is shown, indicating the phosphorylation sites and the FKBP12-binding site. Listed members are from vertebrates unless otherwise indicated: D, *Drosophila*; C, *Caenorhabditis elegans*; X, *Xenopus*. The dendrograms indicate the relative level of amino acid sequence similarity in the kinase domain. Over this domain, ActR-II and Daf-4 have 40% sequence identity, and ActR-IB and Tkv have 60% identity.

BMP receptors (28, 29). Mammalian ALK7 (30, 31) and the related receptor XTrR-I from *Xenopus* (32) have no known ligand. ALK1 (also known as TSR-I) binds TGF- $\beta$  (33) but does so more weakly than T $\beta$ R-I (34) and is not known to mediate a TGF- $\beta$  response (33). ALK2 is commonly referred to as ActR-I because it can bind activin and mediate certain activin responses in cultured cells (28, 33). However, the identity of its physiological ligand is a point of debate. ActR-I can also bind BMP2 and 4 (35, 36), and its mouse homologue can bind TGF- $\beta$  when overexpressed (34, 37). Experiments using *Xenopus* embryo explants have shown that ActR-I/ALK2 mimics the mesoderm ventralizing activity of BMP4 but not the effects of activin or TGF- $\beta$ , which suggests that ActR-I may function as a BMP receptor *in vivo* (39). Based on its expression pattern, it has been suggested that ALK2 may also function as an MIS/AMH type I receptor (38).

In vertebrates, the type II receptor subfamily includes T $\beta$ R-II, BMPR-II, and AMHR, which selectively bind TGF- $\beta$  (40), BMPs (36, 41, 42), and MIS (23, 43), respectively. ActR-II and -IIB bind activins when expressed alone or in concert with activin type I receptors (18, 44, 45). However, ActR-II and -IIB can bind BMPs 2, 4, and 7 and GDF5 in concert with BMP type I receptors (28, 46, 47).

Members of the TGF- $\beta$  receptor family in invertebrates include Thick veins (Tkv) and Saxophone (Sax), which act as a Dpp type I receptors in *Drosophila* (48–51). Tkv most closely resembles the mammalian BMPR-I receptors,

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whereas Sax is somewhat closer to mammalian ALK1 and ALK2. Punt acts as a Dpp type II receptor in concert with Tkv or Sax (52, 53). ATR-I is a *Drosophila* type I receptor closely related to mammalian T $\beta$ R-I and ActR-IB (54). ATR-I can bind human activin, but its real ligand is unknown. In *C. elegans*, larval development is controlled by Daf-1 (21) and Daf-4 (55), which are thought to be type I and II receptors, respectively, for the BMP-like ligand Daf-7 (56).

### Structural Features of the Receptors

**THE EXTRACELLULAR DOMAIN** Type I and II receptors are glycoproteins of approximately 55 kDa and 70 kDa, respectively, with core polypeptides of 500 to 570 amino acids including the signal sequence (18, 26, 40, 44, 57). The extracellular region is relatively short (approximately 150 amino acids), N-glycosylated (58, 59), and contains 10 or more cysteines that may determine the general fold of this region. Three of these cysteines form a characteristic cluster near the transmembrane sequence (54). The spacing of other cysteines varies and is more conserved in type I receptors than in type II receptors.

The transmembrane region and the cytoplasmic juxtamembrane region of type I and II receptors have no singular structural features. However, Ser213 in this region of T $\beta$ R-II is phosphorylated by the receptor kinase in a ligand-independent manner and is required for signaling activity (60). Ser165 in the juxtamembrane region of T $\beta$ R-I is phosphorylated by T $\beta$ R-II in a ligand-dependent manner, and this appears to selectively modulate the intensity of different TGF- $\beta$  responses (61).

**THE GS DOMAIN** A unique feature of type I receptors is a highly conserved 30-amino acid region immediately preceding the protein kinase domain (Figure 2). This region is called the GS domain because of a characteristic SGSGSG sequence it contains (62). Ligand-induced phosphorylation of the serines and threonines in the TTSGSGSG sequence of T $\beta$ R-I by the type II receptor is required for activation of signaling (61–63), and the same happens with the activin type I receptor ActR-IB (64). Immediately following the SGSGSG sequence, all type I receptors have a Leu-Pro motif that serves as a binding site for the immunophilin FKBP12 (65, 66). FKBP12 may act as a negative regulator of the receptor signaling function. The penultimate residue in the GS domain, right at the boundary with the kinase domain, is always a threonine or a glutamine. As shown with T $\beta$ R-I (63) and several other type I receptors (46, 64, 67–69), mutation of this residue to aspartate or glutamate endows the receptor with elevated kinase activity in vitro and constitutive signaling activity in the cell. Thus, the GS domain is a key regulatory region that may control the catalytic activity of the type I receptor kinase or its interaction with substrates.

**THE KINASE DOMAIN** The kinase domain in type I and II receptors conforms to the canonical sequence of a serine/threonine protein kinase domain (18, 24).

Consistent with this, type I receptors have been shown to phosphorylate their substrates—SMAD proteins—on serine residues (68, 70), whereas type II receptors phosphorylate themselves and type I receptors on serine and threonine residues but not tyrosine residues (40, 61–63, 71, 72). Autophosphorylation of T $\beta$ R-II on tyrosine has been observed *in vitro* but not *in vivo* (73).

Conserved residues that in the crystal structure of other protein kinases coordinate ATP phosphate groups are essential for the activity of type I and II receptor kinases. These residues include a universally conserved  $\beta$ 3-strand lysine (27, 74) and G217 in the glycine loop of T $\beta$ R-I (75). The regulatory region known as the T loop in other protein kinases (76) contains two serines in T $\beta$ R-II whose phosphorylation may enhance or inhibit the signaling activity of the receptor (60). A region of interest in the kinase domain of type I receptor kinases is the L45 loop that links two putative  $\beta$  strands. Replacement of the L45 loop in ActR-I with the L45 loop from T $\beta$ R-I allows it to mediate TGF- $\beta$  responses (77). Therefore this region may be involved in substrate recognition.

Type II receptors typically contain a very short C-terminal extension following the kinase domain, whereas type I receptors have essentially no C-terminal extension. Exceptions are the *C. elegans* receptor Daf-4 (55) and an alternative form of human BMPR-II (36, 41, 42, 78) that has long C-terminal extensions of unknown function. The C-terminal extension of T $\beta$ R-II is phosphorylated (61), but its deletion does not impair signaling (79). This is in contrast to the important role that the C-terminal tail plays in signal transduction by tyrosine kinase receptors (80).

**RECEPTOR VARIANTS** Some members of the TGF- $\beta$  receptor family exist in alternative forms. These forms arise from the presence or absence of the following: a 25-amino acid insert following the signal sequence in T $\beta$ R-II (81, 82), a 61-amino acid insert in the same position in AMHR-II (23), two alternative N-terminal regions in Tkv (49, 50), two alternative extracellular juxtamembrane regions in ATR-I (54), small inserts in the extracellular and intracellular juxtamembrane regions of ActR-IIB (44), and a long C-terminal extension in BMPR-II (36, 41, 42, 78). The presence of the extracellular insert in ActR-IIB increases the affinity for activin (44). The functional significance of the other receptor variants is unknown.

## LIGAND-RECEPTOR INTERACTIONS

### *The Binding of Ligand to Signaling Receptors*

**LIGAND STRUCTURE: IMPLICATIONS FOR BINDING** The bioactive forms of TGF- $\beta$  and related factors are dimers held together by hydrophobic interactions and, in most cases, also by an intersubunit disulfide bond (83). Each monomer

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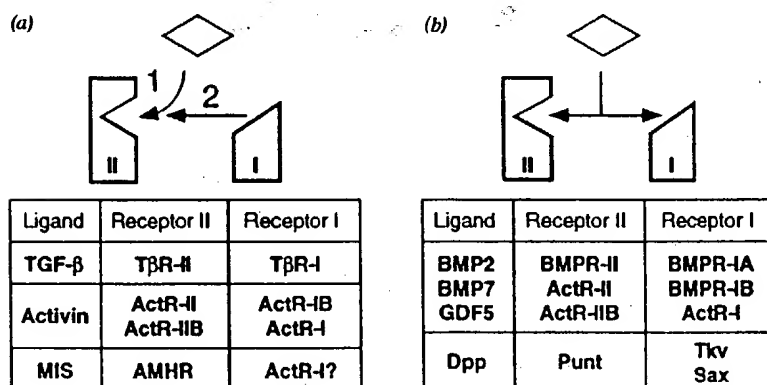
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contains three disulfide bonds interlocked into a tight structure known as the cystine knot (83). Insights into the possible regions of receptor contact are provided by the crystal structures of TGF- $\beta$ 2 (84, 85) and BMP7/OP-1 (86), the solution structure of TGF- $\beta$ 1 (87), and mutational analysis of TGF- $\beta$ 1 and TGF- $\beta$ 2 (88). The dimeric structure of these ligands suggests that they function by bringing together pairs of type I and II receptors, forming heterotetrameric receptor complexes. The pairing of receptors may be further specified by naturally occurring heterodimeric ligands such as TGF- $\beta$ 1.2 (19), TGF- $\beta$ 2.3 (89), and activin AB (90). The recombinant heterodimer BMP-4/7 is more potent in bioassays than BMP4 or BMP7 homodimers (91).

No species specificity has been described in the ligand-receptor interactions of the TGF- $\beta$  system. Dpp receptors and Daf-4 can bind human BMPs (49, 50, 55), *dpp* phenotypes in flies can be rescued with a human *BMP4* transgene (92), and recombinant Dpp can induce endochondral bone formation in mammals (93).

**TWO MODES OF BINDING** TGF- $\beta$  and related factors activate signaling by binding to and bringing together pairs of type I and II receptors. Two general modes of binding ligand have been observed (Figure 3). One mode involves direct binding to the type II receptor and subsequent interaction of this complex with the type I receptor, which, in effect, becomes recruited into the complex. This binding mode is characteristic of TGF- $\beta$  and activin receptors. Type I receptors for these factors can recognize ligand that is bound to the type II



**Figure 3** Two modes of ligand binding: (a) sequential binding, (b) cooperative binding. The ligands that bind according to each mode are listed together with the type I and II receptor combinations that they recognize. TGF, transforming growth factor; BMP, bone morphogenetic protein; GDF, growth and differentiation factor; MIS, Müllerian inhibiting substance.

receptors but not ligand that is free in solution (24, 33, 37, 72). This phenomenon was originally revealed by the receptor phenotype of TGF- $\beta$ -resistant cell mutants (20, 94). TGF- $\beta$ 1 can bind to T $\beta$ R-II in cell mutants lacking T $\beta$ R-I but cannot bind to T $\beta$ R-I in mutants lacking T $\beta$ R-II. Restoration of T $\beta$ R-II ligand-binding function, either by somatic fusion of a T $\beta$ R-I defective cell with a T $\beta$ R-II defective cell (95) or by transfection of a T $\beta$ R-II cDNA (74), restores TGF- $\beta$  binding to T $\beta$ R-I. Furthermore, recombinant T $\beta$ R-II binds TGF- $\beta$ 1 in solution (96-98). Like T $\beta$ R-II, the type II receptors ActR-II (18), ActR-IIB (44, 45), and AMHR (23) also bind ligand when transfected in the absence of a type I receptor. Indeed, the original cloning of T $\beta$ R-II (40) and ActR-II (18) was based on the ability of these receptors to bind ligand when overexpressed in COS cells.

The second binding mode is typical of BMP receptors and is cooperative, involving type I and II receptors that bind ligand with high affinity when expressed together but low affinity when expressed separately (36, 41, 42, 47) (Figure 3). Thus, BMPs 2, 4, and 7 and GDF5 bind weakly to the type II receptor BMPR-II expressed alone (36, 41, 42, 47) and to the type I receptors BMPR-IA or BMPR-IB expressed alone (29, 35) or in solution (99). ActR-II and -IIB are bona fide activin receptors that on their own bind BMP poorly if at all. However, ActR-II and -IIB can bind BMPs 2 and 7 in cooperation with BMPR-IA or BMPR-IB (28). This interaction mirrors what is observed with the Dpp receptor system. The Dpp type II receptor Punt, which is more closely related to ActR-II and -IIB than it is to BMPR-II, can recognize human activin (100). However, genetic evidence indicates that Punt acts as a Dpp receptor, and this evidence led to the finding that Punt binds Dpp or BMP poorly on its own but well in the presence of Tkv or Sax (52).

#### *Accessory Receptors: Betaglycan and Endoglin*

The original search for cell surface TGF- $\beta$ -binding proteins using ligand cross-linking methods revealed the existence of binding proteins that were classified, according to their molecular weight, as type I and type II receptors (reviewed above) and type III receptors (19). Type III receptors detected by ligand cross-linking turned out to correspond to either one of two related proteins, betaglycan or endoglin (101-103). The evidence to date suggests that type III receptors do not have an intrinsic signaling function but regulate TGF- $\beta$  access to the signaling receptors. There is no concrete evidence for type III receptors for other TGF- $\beta$  family members.

**BETAGLYCAN** Betaglycan is a membrane-anchored proteoglycan (58, 104) with an 853-amino acid core protein (101, 102) that carries heparan sulfate and chondroitin sulfate glycosaminoglycan (GAG) chains attached to Ser535

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and Ser546 (105, 106). In the cell, betaglycan appears to exist as noncolalent homodimers (107). GAG chains are not required for betaglycan to reach the cell surface or to bind TGF- $\beta$ , as revealed by studies using cell mutants defective in GAG synthesis (108) and betaglycan mutants defective in GAG attachment sites (105, 106). However, GAG chains of betaglycan can bind fibroblast growth factor (109). The cytoplasmic region of betaglycan is short (43 amino acids) and lacks any discernible signaling motif (101, 102). This region is not required for the TGF- $\beta$  binding and presentation functions of betaglycan (105), and its function remains unknown. The highest level of sequence similarity between betaglycan and endoglin is found in the cytoplasmic and transmembrane domains (110).

TGF- $\beta$  binding activity has been demonstrated in separate N-terminal and C-terminal domains of the extracellular region of betaglycan (105, 111, 112). The N-terminal domain has sequence similarity to the corresponding region in endoglin (110). The C-terminal extracellular domain contains the GAG attachment sites (105) and shows sequence similarity to a region of the major urinary protein uromodulin, the pancreatic zymogen granule protein GP-2, and the sperm receptors Zp2 and Zp3 (113). The entire extracellular region of betaglycan may be shed into the medium (114), and it may act as a TGF- $\beta$  antagonist, inhibiting binding to membrane receptors (105).

Betaglycan binds all three TGF- $\beta$  forms with high affinity (115, 116) and facilitates TGF- $\beta$  binding to the type II receptor (102, 117), forming a betaglycan/TGF- $\beta$ /T $\beta$ R-II complex in the process (117, 118). The role of betaglycan as a facilitator of TGF- $\beta$  binding to the signaling receptors is most evident with TGF- $\beta$ 2. Like TGF- $\beta$ 1 and - $\beta$ 3, TGF- $\beta$ 2 signals through T $\beta$ R-I and T $\beta$ R-II (74, 95). However, unlike them, TGF- $\beta$ 2 has low intrinsic affinity for T $\beta$ R-II (116) and is less potent than TGF- $\beta$ 1 in hematopoietic progenitor cells (119), myoblasts (117), and endothelial cells (116) that lack betaglycan. Transfection of betaglycan augments TGF- $\beta$ 2 binding and activity in these cells (117, 120). The ability of betaglycan to equalize the potency of all three TGF- $\beta$  forms raises the possibility that betaglycan may not only concentrate TGF- $\beta$  at the cell surface but may also stabilize TGF- $\beta$ s in a conformation optimal for binding to the signaling receptors.

**ENDOGLIN** Endoglin is a cell surface molecule expressed at high levels in endothelial cells and at lower levels in monocytes, erythroid precursors, and other cell types (103, 121). Two splice variants of the cytoplasmic region give rise to human endoglin forms of 625 and 658 amino acids (122), each forming disulfide-linked dimers (103, 122). The sequence similarity between endoglin and betaglycan prompted an analysis of TGF- $\beta$  binding to endoglin. This revealed that endoglin binds TGF- $\beta$ 1 and - $\beta$ 3, but unlike betaglycan, it does not

bind TGF- $\beta$ 2 (110). As with betaglycan, complexes between endoglin and TGF- $\beta$  receptors have been observed (123).

However, the role of endoglin in TGF- $\beta$  binding to signaling receptors is unclear. The TGF- $\beta$  binding activity of endoglin is limited compared to that of betaglycan and is increased by coexpression of T $\beta$ R-II. In fact, endoglin overexpression can diminish rather than enhance TGF- $\beta$  responses in monocytes (121). As mentioned below, mutations in *endoglin* and *ALK1* give rise to similar human disorders (124–126). Endoglin and ALK1 therefore might act in the same pathway, with endoglin facilitating ligand binding to ALK1. Given the weak TGF- $\beta$ -binding activities of both receptors, the common endoglin and ALK1 ligand may not have been identified yet.

#### *Latent Ligands and Soluble Inhibitory Proteins*

The activity of TGF- $\beta$  and related factors is negatively regulated by various soluble proteins that prevent their interaction with membrane receptors (see Figure 6).

**THE LATENT TGF- $\beta$  COMPLEX** Like all other members of its family, TGF- $\beta$  is synthesized as the C-terminal domain of a precursor form that is cleaved before secretion from the cell (127, 128). However, the TGF- $\beta$  propeptide, which is referred to as the latency associated peptide (LAP), remains noncovalently bound to TGF- $\beta$  after secretion, retaining TGF- $\beta$  in a latent form that cannot bind to betaglycan or the signaling receptors (129). Most cell types secrete TGF- $\beta$  in this biologically inert form (12). Although LAP may be destroyed in the process of TGF- $\beta$  activation, recombinant LAP retains TGF- $\beta$  masking ability, and its injection in mice can inhibit endogenous TGF- $\beta$ 1 action (130).

A third component of the latent TGF- $\beta$  complex is a large secretory glycoprotein known as latent TGF- $\beta$ -binding protein (LTBP), which is disulfide-linked to LAP (131). LTBP is not required for the latency of the TGF- $\beta$  complex but is implicated in the secretion, storage in the extracellular matrix, and eventual activation of this complex (131). LTBP comprises several forms generated from two genes and by alternative splicing: LTBP-1 in short and long forms (132) and LTBP-2 (133). Structurally, LTBPs contain a core of epidermal growth factor (EGF) repeats and eight-cysteine motifs organized in a fashion resembling fibrillin-1 and -2—two microfibrillar proteins whose mutations cause Marfan's syndrome and congenital contractural arachnodactyly, respectively (133). Like fibrillins, LTBP undergoes cross-linking by transglutaminases, forms fibrillar structures, and associates tightly with the extracellular matrix in mesenchymal and endothelial cells (134).

In tissue culture, LTBP associated with the extracellular matrix mediates storage of latent TGF- $\beta$  and facilitates its activation (134, 135). Latent TGF- $\beta$

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can be activated in vitro by acid, alkali, heat, limited proteolysis, or incubation by glycosidases (131). In tissue culture, activation of latent TGF- $\beta$  may involve a combination of steps including the following: LAP proteolysis, binding to the mannose 6-phosphate/type II insulin-like growth factor receptor (Man6P/IGFR-II) via a mannose 6-phosphate group in LAP, cell-cell interactions between endothelial and vascular smooth-muscle cells, and binding to thrombospondin (131, 134–136). However, the physiological activation mechanism or mechanisms remain to be defined.

**THE INHIBIN  $\alpha$  CHAIN** Inhibin is the name given to heterodimers between the inhibin  $\alpha$  chain and an inhibin/activin  $\beta$  chain (137). Inhibin was identified as an inhibitor of follicle-stimulating hormone (FSH) production in pituitary cultures (9). The subsequent identification of activins as  $\beta$ -chain dimers with biological activities opposite those of inhibin led to the idea that inhibins and activins are mutual antagonists (9). Because inhibin can compete for binding to the activin receptors ActR-II and -IIB (18, 44), it might antagonize activin by binding to its receptors without triggering signaling, either by failing to recruit type I receptors or by failing to achieve their activation (138, 139). The inhibin  $\alpha$  chain therefore can be regarded as an inhibitor that functions by associating with  $\beta$  chains generating activin receptor antagonists. However, some effects of inhibin could be mediated by as yet unidentified inhibin receptors.

**THE ACTIVIN INHIBITOR FOLLISTATIN** Follistatin is a soluble glycoprotein originally identified for its ability to inhibit pituitary FSH production (140) and later found to bind activin (141). Follistatin prevents activin binding to cell surface receptors (142). Paracrine as well as endocrine anti-activin effects of follistatin have been demonstrated in diverse tissues in mammals and *Xenopus* (140, 143–145). Follistatin can also bind to BMP-7, albeit with lower affinity than to activin (28), and may antagonize BMP signaling in vivo (145). Mammalian follistatin exists in forms of 288 and 315 amino acids generated by alternative splicing (146, 147). Follistatin is expressed in diverse mammalian tissues during development and in the adult (148–150) and in the Spemann's organizer in *Xenopus* embryos (145).

**THE BMP INHIBITORS NOGGIN AND CHORDIN/SOG** The Spemann's organizer, a signaling center at the dorsal lip of the *Xenopus* gastrula blastopore, secretes BMP antagonists—noggin and chordin—which allow neighboring cells to develop as neural or dorsal mesoderm rather than epidermal or ventral mesoderm tissues (151, 152). Although noggin and chordin are of unrelated primary structure, both bind BMP4 (but not TGF- $\beta$  or activin), preventing its interaction with cell surface receptors (151, 152). Noggin, a 222-amino acid polypeptide that is secreted as a homodimer, was the first such antagonist to be identified

(153). In the mouse, a noggin homologue is expressed in specific regions of the nervous system (154). Chordin has four cysteine-rich repeats similar to those found in thrombospondin,  $\alpha 1$  procollagen, and von Willebrand factor (155). In *Drosophila*, the short gastrulation gene product, Sog, is the structural and functional homologue of chordin (156–158) and prevents Dpp from signaling through its receptors (159). The structural differences between noggin and chordin may result in different abilities to diffuse from their source, interact with extracellular matrix, and/or recognize different members of the large and complex BMP subgroup.

### MECHANISM OF RECEPTOR ACTIVATION

Studies on the mechanism of activation of serine/threonine kinase receptors have centered on TGF- $\beta$  receptors. However, to the extent that these studies have been replicated with activin and BMP receptors, the same basic activation mechanism appears to operate in these receptors as well.

#### *The Basal State*

**BASAL PHOSPHORYLATION** The TGF- $\beta$  type I receptor, T $\beta$ R-I, is not phosphorylated in the basal state (62), but T $\beta$ R-II, ActR-II, and ActR-IIB are (40, 62, 64, 71, 160). Their basal phosphorylation is on serine residues and is partially retained in kinase-defective receptor mutants (62, 64). Some of the sites involved are in the C-terminal tail. Their functional significance is unclear. In one study, deletion of this entire region had no detectable effect on receptor signaling (79). Phosphorylation of other sites within T $\beta$ R-II is dependent, directly or indirectly, on the activity of the receptor kinase (62, 64). In T $\beta$ R-II, these sites include a serine in the juxtamembrane region and serines in the T-loop region of the kinase domain, and their phosphorylation modulates the signaling activity of T $\beta$ R-II (60). What regulates the phosphorylation of these sites is not known.

**BASAL RECEPTOR OLIGOMERIZATION** The oligomeric state of endogenous TGF- $\beta$  receptors is not known, but studies with transfected epitope-tagged receptors indicate that T $\beta$ R-II can form ligand-independent homo-oligomers (107, 161). These complexes are thought to prime the formation of the heteromeric T $\beta$ R-I/T $\beta$ R-II receptor complex upon ligand binding.

Type I and II receptors have intrinsic affinity for each other, as manifested by the spontaneous association of T $\beta$ R-I and T $\beta$ R-II when overexpressed in insect cells or coinubated in vitro as recombinant proteins (96). In the absence of ligand, T $\beta$ R-I and T $\beta$ R-II (162) or ActR-IB and ActR-IIB (64) can form active complexes when overexpressed in mammalian cells. This interaction is mediated, at least in part, by the cytoplasmic regions because these



regions interact in a yeast two-hybrid system (36, 78, 96, 160). However, in transfected cells expressing moderate levels of TGF- $\beta$  receptors (62) or activin receptors (138), the heteromeric receptor complex and, in particular, the phosphorylation and activation of the type I receptor are highly dependent on ligand binding.

**FKBP12 BINDING** The cytoplasmic domain of diverse type I receptors interacts with FKBP12 in yeast (36, 163, 164) and mammalian cells (66, 165, 166). FKBP12 is an abundant 12-kDa cytosolic protein with *cis-trans* peptidyl-prolyl isomerase (rotamase) activity (167). FKBP12 binds different proteins, some on its own and some as a target of various natural or synthetic immunosuppressants. On its own, FKBP12 binds to the ryanodine receptor and the inositol 1,4,5-triphosphate receptor, stabilizing the calcium channeling activity of these proteins (168, 169). In complex with the drug FK506, FKBP12 binds calcineurin, inhibiting calcineurin's phosphatase activity and thus its ability to activate the transcription factor NF-AT in the T-cell receptor signal transduction pathway (170). In complex with rapamycin, FKBP12 binds FRAP/RAFT, inhibiting its activity as a kinase in mitogenic signal transduction (171, 172).

FKBP12 binding to T $\beta$ R-I inhibits TGF- $\beta$  signaling (66, 166) by inhibiting T $\beta$ R-I phosphorylation by T $\beta$ R-II within the oligomeric receptor complex (66). FKBP12-receptor interaction is mediated by the active site of FKBP12 (66, 166) and a conserved Leu-Pro motif adjacent to the phosphorylation sites in the GS domain of the receptor (65, 66) (Figure 2). FKBP12 binds to the TGF- $\beta$  type I receptor in the basal state and appears to be released upon TGF- $\beta$ -induced formation of the receptor complex (66, 166). Mutant T $\beta$ R-I receptors defective in FKBP12 binding have elevated basal signaling activity but normal signaling activity in the presence of ligand (66). Therefore, one function of FKBP12 may be to guard against spurious activation of TGF- $\beta$  signaling by ligand-independent encounters of type I and II receptors.

**OTHER RECEPTOR-BINDING PROTEINS** TRIP-1 was identified as a T $\beta$ R-II-interacting protein in a yeast two-hybrid screen (173). TRIP-1 contains several WD domains that may mediate protein-protein interactions, but the role of TRIP-1 is unknown. The interaction of TRIP-1 and T $\beta$ R-II in mammalian cells is independent of ligand, requires the kinase activity of the receptor, and causes TRIP-1 phosphorylation (173).

The T $\beta$ R-I cytoplasmic domain can interact with the farnesyl transferase- $\alpha$  subunit when both components are overexpressed in yeast or mammalian cells (164, 174, 175). It has been suggested that TGF- $\beta$  may signal by regulating farnesyl transferase activity (174). However, this notion is controversial because the TGF- $\beta$  receptor does not associate with the farnesyl transferase holoenzyme

(175). Furthermore, cells do not show a change in farnesyl transferase activity or in the farnesylation pattern of specific proteins in response to TGF- $\beta$  (175).

### The Activated State

**RECEPTOR COMPLEX FORMATION** Signals emanate from a TGF- $\beta$  type I receptor when it is phosphorylated by its activator, the type II receptor. As first shown with TGF- $\beta$  receptors (74), ligand binding induces the formation of a heteromeric complex of type I and II receptors (24, 25, 27, 33, 36, 41, 62, 64, 74, 138, 176) (Figure 1). Given the dimeric nature of the ligands, each monomer might contact one type I receptor and one type II receptor, thereby generating a heterotetrameric receptor complex. Indeed, that the ligand-induced heteromeric complex contains two or more type I receptor subunits and two or more type II receptor subunits is suggested by analysis of TGF- $\beta$  receptor complexes on two-dimensional gel electrophoresis (25), coprecipitation of receptors containing distinct epitope tags (75), and genetic complementation between mutant type I receptors (75). The TGF- $\beta$  receptor complex is extremely stable upon solubilization, resisting dissociation by ionic detergents and chaotropic agents (62). Formation of this complex is required for signaling. Using chimeric receptor constructs containing T $\beta$ R-I and T $\beta$ R-II kinase domains in different configurations, signaling is achieved only when type I and II receptor kinase domains are brought together (177–179).

**TYPE II RECEPTOR KINASE ACTIVITY** Ligand binding does not increase the overall phosphorylation of the type II receptors T $\beta$ R-II, ActR-II, or ActR-IIB or their kinase activity *in vitro* (62, 64, 71, 162). Thus, type II receptors might be constitutively active kinases that require the ligand to interact with the type I receptor as a substrate. One caveat with this notion is that these studies have been done with moderately overexpressed receptors. It remains possible that type II receptors expressed at endogenous levels may undergo a ligand-induced increase in kinase activity. In any case, even when moderately overexpressed, type II receptors require ligand to phosphorylate their substrates, type I receptors.

**TRANSPHOSPHORYLATION** Formation of the ligand-induced receptor complex rapidly leads to phosphorylation of the type I receptor (Figure 1), as demonstrated with TGF- $\beta$  (62, 162) and activin receptors (64, 180). This phosphorylation is catalyzed by the type II receptor, as shown by coexpression of wild-type and kinase-defective type I and II receptors in different combinations (62, 64, 162). T $\beta$ R-I is phosphorylated by T $\beta$ R-II at serine and threonine residues in the sequence TTSGSGSGLP of the GS domain (61–63) (Figure 2), and similar sites are phosphorylated in ActR-IB by activin type II receptors (64). In addition to these sites, T $\beta$ R-II mediates phosphorylation of Ser165 in

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the juxtamembrane region of T $\beta$ R-I—a phosphorylation that may positively or negatively affect various TGF- $\beta$  responses (61). T $\beta$ R-I can catalyze its own phosphorylation in vitro, but there is no evidence that this occurs in vivo (63, 72, 75).

### *Signal Flow in the Receptor Complex*

The events that transduce TGF- $\beta$  signals start with type II receptor-mediated activation of the type I receptor. This receptor then phosphorylates and activates SMAD proteins, which carry the signal to the nucleus. This model is based on several lines of evidence. Mammalian cell mutants defective in either T $\beta$ R-I (94) or T $\beta$ R-II (20) lack a wide range of TGF- $\beta$  responses. These responses are recovered in somatic hybrids between these two mutant phenotypes (95) or by transfection of the corresponding wild-type receptor (24, 72, 74). Work in *Drosophila* provides additional genetic evidence that Dpp signaling requires both type I and type II receptors (52, 53). Phosphorylation of serines and threonines in the GS domain of T $\beta$ R-I is required for signaling (61–63). Alanine or valine mutations of any of these sites in T $\beta$ R-I does not prevent phosphorylation of the other sites or receptor activation (63). However, mutation of three or more of these sites to alanine, valine, or acidic residues in T $\beta$ R-I or ActR-IB prevents phosphorylation and signal transduction (63, 64, 180). Signaling is also inhibited when T $\beta$ R-I phosphorylation is prevented by mutations in T $\beta$ R-I or T $\beta$ R-II that impair recognition of T $\beta$ R-I as a substrate (75, 181), or by FKBP12 binding to the Leu-Pro motif in the GS domain (66).

A role of the type I receptor as the downstream signaling component in the receptor complex was originally inferred from the observation that the kinase activity of T $\beta$ R-I is required for signal transduction and yet its substrate is neither T $\beta$ R-I nor T $\beta$ R-II (62). It was also shown that different type I receptors determine distinct responses to the same agonist (27, 182). Key evidence for a downstream role of the type I receptor was provided by the fact that hyperactive forms of T $\beta$ R-I (63), ActR-IB (64), BMPR-IA and -IB (46, 68, 69), and Tkv (46, 67), generated by a mutation in the GS domain, have constitutive signaling activity in vivo. Signaling by hyperactive T $\beta$ R-I also has been demonstrated in T $\beta$ R-II-defective cells (63). The ability of purified BMP type I receptor to directly phosphorylate the activation sites of Smad1 in vitro (68) provides compelling evidence that in TGF- $\beta$  receptor complexes, the signal flows from the type II receptor to the type I receptor and on to SMADs.

It is not clear whether activation of the type I receptor is based on an increase in its kinase activity, the appearance of substrate binding sites, or a combination of these two mechanisms. The hyperactive form T $\beta$ R-I(T204D) has higher autokinase activity in vitro (63), suggesting that receptor activation may involve an increase in intrinsic kinase activity. On the other hand, it has been shown that

T $\beta$ R-I activation results in Smad2 binding to the receptor complex (70, 183), suggesting that receptor activation may result in the generation of substrate docking sites.

In theory, the type II receptor could also signal independently of the type I receptor by phosphorylating other, as yet unidentified, signal-transducing substrates. However, no TGF- $\beta$  responses have been described in cells lacking type I receptors. Overexpression of dominant-negative T $\beta$ R-II receptor constructs can eliminate all TGF- $\beta$  responses tested (79, 184) or only part of the TGF- $\beta$  responses tested (185), depending on the assay conditions. Responses requiring a low level of signaling activity may be triggered by a residual level of activity in cells expressing dominant-negative receptors.

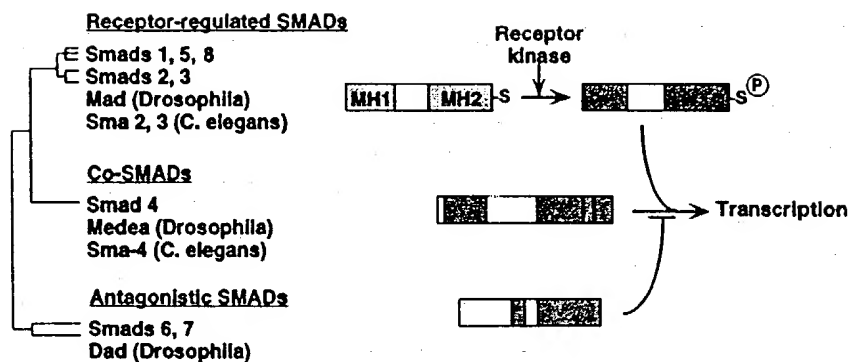
## SMAD PROTEINS

The proteins of the SMAD family are the first identified substrates of type I receptor kinases and play a central role in the transduction of receptor signals to target genes in the nucleus (Figure 1).

### *SMADs as Mediators of TGF- $\beta$ Signaling*

The founding member of the SMAD family is the product of the *Drosophila* gene *Mad* (*mothers against dpp*) (186). *Mad* was identified in a genetic screen for mutations that exacerbate the effect of weak *dpp* alleles (187), and its discovery led to the identification of many related genes in nematodes and vertebrates. Three *Mad* homologues were identified in *C. elegans* and called  *sma-2*, *-3*, and *-4* because their mutation causes small body size (188). Shortly thereafter, many homologues were described in vertebrates and named SMADs (for SMA/MAD related). *DPC4* (for "deleted in pancreatic carcinoma locus 4"), a gene frequently mutated or deleted in pancreatic cancer (189), also referred to as *Smad4*, was one of the first reported human SMADs. Human, mouse, and/or frog Smads 1–8 were cloned by screening EST (expressed sequence tag) databases or cDNA libraries for *Mad* homologues (46, 183, 190–198). Smad2 was independently identified in a cDNA expression cloning screen for inducers of mesoderm formation in *Xenopus* embryos (199). Smads 6 and 7 were identified as shear stress-induced genes in endothelial cells (200).

Initial evidence that SMADs function downstream of TGF- $\beta$  receptors was provided by the ability of *Mad* mutations to inhibit signaling by a hyperactive Tkv receptor construct (46, 67). The most compelling evidence came from the observation that in response to TGF- $\beta$  and related agonists, SMADs are phosphorylated (46, 183, 192–194, 201), accumulate in the nucleus (46, 191, 199), and become transcriptionally active (191). This body of evidence placed SMADs squarely downstream of TGF- $\beta$  receptors.



**Figure 4** The SMAD family. Listed members are from vertebrates unless otherwise indicated. Vertebrate SMADs are highly conserved between human and *Xenopus*. The dendrogram indicates the relative level of amino acid sequence identity between vertebrate SMADs. The highly conserved MH1 and MH2 domains are indicated. Receptor-regulated SMADs are directly phosphorylated by TGF- $\beta$  family type I receptors, and this phosphorylation allows association with a collaborating SMAD (co-SMAD). Antagonistic SMADs inhibit this SMAD activation process.

### SMAD Subfamilies and Their Functions

Based on structural and functional considerations, SMADs fall into three subfamilies (Figure 4): (a) SMADs that are direct substrates of TGF- $\beta$  family receptor kinases, (b) SMADs that participate in signaling by associating with these receptor-regulated SMADs, and (c) antagonistic SMADs that inhibit the signaling function of the other two groups.

Among the receptor-regulated SMADs, Smad1 and presumably its close homologues Smad5 and Smad8 are substrates for BMPR-I (68) and mediators of BMP signals (46, 190, 191, 202, 203, 203a). Smads 2 and 3 are T $\beta$ R-I substrates (70, 183) and mediators of TGF- $\beta$  and activin signals (190, 193, 195, 199, 201, 204). When overexpressed in *Xenopus* early embryos, Smad1 mimics the ability of BMP4 to ventralize mesoderm (190, 191, 202), whereas Smad2 mimics dorsal mesoderm induction and axis formation by activin (190, 199). In mammalian epithelial cells, Smads 2 and 3 mediate growth inhibition and transcriptional activation of TGF- $\beta$  and activin reporter genes (183, 201). Mad and Sma's 2 and 3 also belong to this subfamily; they act as mediators of Dpp receptor signals (205) and Daf-4 signals (188), respectively.

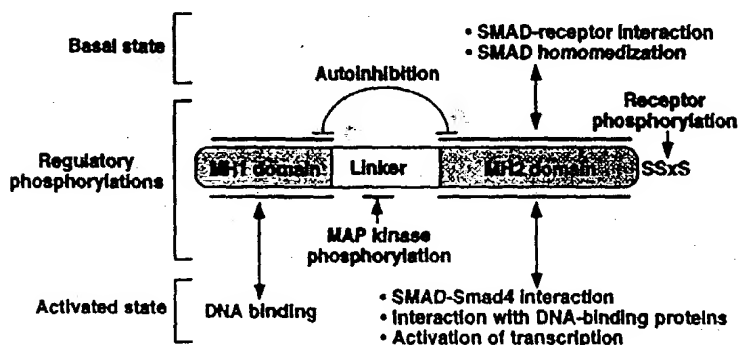
Signaling by receptor-regulated SMADs requires the participation of a collaborating SMAD. The only known member of this group in vertebrates is Smad4. Smad4 associates with receptor-regulated SMADs when these become phosphorylated by the corresponding receptors (68, 183, 201, 206). Although Smad4 is similar to the receptor-regulated SMADs in overall structure,

it normally is not phosphorylated in response to agonists. Smad4 is required for Smad2- or Smad3-dependent growth inhibitory responses in mammalian cells, and a dominant-negative Smad4 construct interferes with Smad1 and Smad2 signaling in frog embryos and mammalian cells (183, 201). Smad4, therefore, participates in TGF- $\beta$ , activin, and BMP signaling pathways as a shared partner of receptor-regulated SMADs. The *Medea* (206a-c) and *Sma-4* (188) gene products from *Drosophila* and nematode are close homologues of Smad4, and they may fulfill a similar function in these organisms.

Human Smads 6 and 7 and *Drosophila* Dad are a subfamily of structurally divergent SMADs whose only known activity is to inhibit the signaling function of receptor-activated SMADs. Smad6 preferentially inhibits BMP signaling (196, 207), Smad7 can inhibit TGF- $\beta$  and BMP signaling (197, 208), and Dad inhibits Mad signaling (209). Additional SMADs have been identified in nematode, but their functional properties are complex, as inferred from genetic analysis (210).

### Structural Features of SMADs

**THE MH1 DOMAIN** SMAD proteins contain highly conserved N-terminal and C-terminal domains (referred to as N and C domains, or MH1 and MH2 domains, respectively) and an intervening linker region that is of variable length and sequence (Figure 5). The MH1 domain has approximately 130 amino acids



**Figure 5** SMAD domains and their functions. In the basal state, SMADs form homo-oligomers and remain in an inactive state through an interaction between the MH1 and MH2 domains. Receptor-regulated SMADs interact with activated type I receptors via the MH2 domain and become activated by receptor-mediated phosphorylation at the C-terminal SS(V/M)S motif. In the activated state, SMADs associate with Smad4 and with DNA-binding proteins via the MH2 domain. The MH1 domain of some SMADs also participates in DNA binding, and the MH2 domain participates in transcriptional activation. MAP kinases phosphorylate some SMADs in the linker region, inhibiting SMAD accumulation in the nucleus.

and is highly conserved in receptor-regulated SMADs and Smad4 but not in inhibitory SMADs. In the basal state, the MH1 domain inhibits the transcriptional (191) and biological (199) activities of the MH2 domain. This inhibitory effect is likely due to the interaction between these two domains. Indeed, the MH1 domains of Smads 2 and 4 can physically interact with the respective MH2 domains, and overexpression of either MH1 domain as a separate protein can prevent the TGF- $\beta$ -induced association of Smad2 and Smad4 MH2 domains (206).

The MH1 domain does not have a purely inhibitory function because it has DNA-binding activity in the activated state. The DNA-binding activity of the Mad MH1 domain is required for Dpp-induced activation of an enhancer within the *vestigial* wing-patterning gene (211). Likewise, the Smad4 MH1 domain contributes to the DNA-binding activity of a Smad2-Smad4 transcriptional complex (212). The DNA-binding activity of the Mad MH1 domain is inhibited by the presence of the MH2 domain (211), suggesting that the MH1 and MH2 domains may inhibit each other's function in the basal state. The contribution of the MH1 domains to the DNA-binding affinity and specificity of SMAD transcriptional complexes may vary depending on the particular target gene.

**THE MH2 DOMAIN** This domain contains receptor phosphorylation sites (in receptor-regulated SMADs) (68, 70), has effector function (191, 199), and is involved in several important protein-protein interactions (Figure 5). The canonical MH2 domain is about 200 amino acids long and contains a characteristic insert in the case of Smad4 and Sma-4 (183). Interactions between MH2 domains support the homo-oligomeric complexes that SMADs from all three subfamilies form in the basal state (201, 206, 207, 213, 214). The MH2 domains also mediate the association of receptor-regulated SMADs with type I receptors (70), with Smad4 upon receptor-mediated phosphorylation (206), and with DNA-binding factors (212, 215) (see below). The Smad2 MH2 domain is biologically active in frog mesoderm induction assays (199), and when fused to the DNA-binding domain of GAL4, the MH2 domains of Smad1 and Smad2 display agonist-independent transcriptional activity (191, 212). Smads 1 and 2 require the presence of the Smad4 MH2 domain to activate transcription (212). In the case of antagonistic SMADs, the MH2 domain is sufficient for their inhibitory effect (200, 207).

The crystal structure of the Smad4 MH2 domain has provided insights into the basis for some of these interactions (214). The Smad4 MH2 domain forms a homotrimer in the crystals, and Smad4 forms a trimer in solution. Each monomer consists of a  $\beta$ -sandwich core flanked by three  $\alpha$ -helices in a bundle on one side and several loops and an  $\alpha$ -helix on the other side. The trimer interfaces are formed by extensive contacts between the three-helix bundle of one monomer and the loops on the adjacent monomer. Tumor-derived mutations



in these interfaces destabilize and inactivate the homotrimer (see below). The trimer has the shape of a disc with the linker region emerging from one face. A loop referred to as the L3 loop protrudes from each monomer on the other face, and an  $\alpha$ -helix referred to as helix-2 protrudes from each monomer on the edge of the disc. The L3 loops and the helix-2 may be sites for interaction with other proteins. Indeed, mutations in the L3 loop prevent Smad2 from interacting with the TGF- $\beta$  receptor (217) and Smad4 from interacting with Smad2 (214). Based on sequence similarities, the overall structure of the MH2 domain is likely to be conserved in the other SMADs. Smads 6 and 7 lack the region corresponding to the third helix of the bundle, so they may form a different type of monomer-monomer interface (207).

**THE LINKER REGION** The linker region is highly variable in size and sequence. This region contributes to the formation of SMAD homo-oligomers (206, 213). In receptor-regulated SMADs, the linker region contains MAP-kinase phosphorylation sites (216). As discussed below, phosphorylation of these sites in response to MAP-kinase activation inhibits nuclear translocation of SMADs.

## SIGNALING THROUGH SMADs

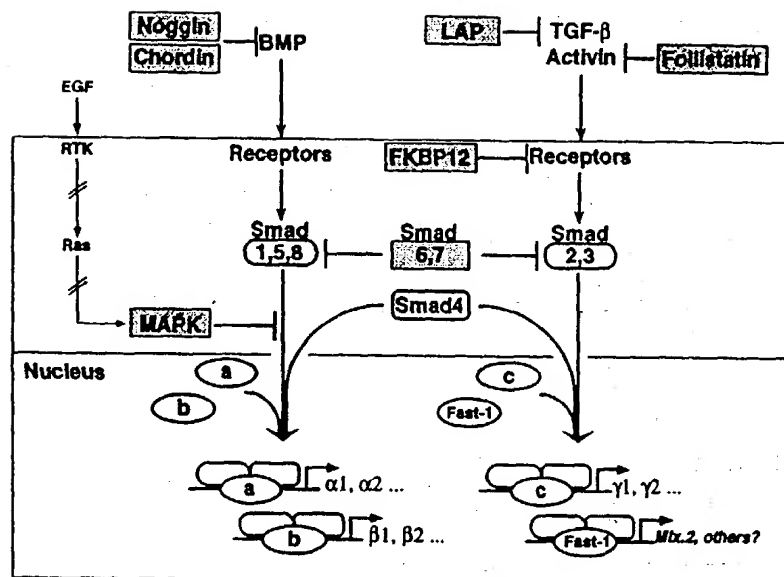
In the basal state, SMADs exist as homo-oligomers that reside in the cytoplasm (Figures 1 and 6). Upon ligand activation of the receptor complex, the type I receptor kinase phosphorylates specific SMADs, which then form a complex with Smad4 and move into the nucleus. In the nucleus, these complexes, either alone or in association with a DNA-binding subunit, activate target genes by binding to specific promoter elements.

### *SMADs as Receptor Substrates*

**PHOSPHORYLATION SITES** SMADs are serine-phosphorylated in response to agonists, as shown with Smad1 in response to BMP2 or 4 (46, 68), Smad2 in response to TGF- $\beta$  or activin (70, 193, 201), and Smad3 in response to TGF- $\beta$  (183, 204). Although the kinetics of this phosphorylation are relatively slow ( $t_{1/2} \sim 5$  min) when transfected SMADs are used, evidence shows that SMADs are direct substrates of the receptors. Smad1 is phosphorylated by highly purified, bacterially expressed BMPR-I kinase domain (68), Smad2 by immunoprecipitated TGF- $\beta$  receptor complexes (70), and Smad3 by a T $\beta$ R-I kinase preparation (183).

In vitro and in vivo, receptor-mediated phosphorylation occurs at serines in the C-terminal motif SS(V/M)S of Smad1 (68) or Smad2 (68, 70). This motif is also present in Smads 3, 5, and 8; *Drosophila* Mad; and *C. elegans* Smas-2 and -3. However, it is not present in the Smad4 subfamily or the inhibitory SMADs. This is consistent with the commonly observed lack of





**Figure 6** A model for generation of diverse gene responses by the SMAD system and their negative regulation. Smads 1, 5, and 8 are BMP receptor substrates and mediators of BMP gene response, whereas Smads 2 and 3 are substrates and mediators of TGF- $\beta$  and activin receptors. Hypothetically each SMAD complex associates with different sequence-specific DNA-binding factors, of which Fast-1 is the first known example, and activates a distinct set of target genes. Negative regulation is provided by growth factor-sequestering proteins, FKBP12 binding to type I receptors, antagonistic SMADs, and SMAD phosphorylation by MAP kinases (in gray boxes). LAP, latency associated peptide.

agonist-induced phosphorylation in Smads 4, 6, and 7 (183, 196, 197, 201, 207, 208). Mutation of the serines in this sequence inhibits receptor-mediated phosphorylation of Smads 1 and 2 in vivo and in vitro and their association with Smad4 (68), accumulation in the nucleus (68, 70), interaction with DNA-binding proteins (212), and mediation of transcriptional responses (68, 70). Therefore, phosphorylation of this motif is required for SMAD activation.

**SMAD-RECEPTOR ASSOCIATION** Smad2 and Smad3 become transiently and selectively associated with the activated TGF- $\beta$  receptor complex (70, 183, 217). This interaction is required for Smad2 phosphorylation because docking-defective Smad2 mutants are not phosphorylated in response to TGF- $\beta$  (70, 217). The Smad2 phosphorylation sites themselves along with the adjacent sequence in the 11-amino acid C-tail region are not required for this interaction (217). In fact, phosphorylation of these sites appears to facilitate Smad2 dissociation from

the receptor, as either phosphorylation-defective Smad2 mutants or a kinase-defective TGF- $\beta$  type I receptor mutant enhances SMAD-receptor association (70, 217). The transient nature of the SMAD-receptor interaction is consistent with the role of SMADs as carriers of receptor signals into the nucleus.

Mutational analysis of Smads 1 and 2 has identified their L3 loops as critical determinants of receptor docking interactions (217). The integrity of the L3 loop is necessary for interaction with the receptor and sufficient to dictate the specificity of this interaction. The L3-loop sequence is invariant among TGF- $\beta$ -activated Smads (Smads 2 and 3) and BMP-activated Smads (Smads 1, 5, 9 and Mad) but differs at two positions between these two groups. Switching these two amino acids switches Smad1 and Smad2 activation by BMP and TGF- $\beta$ , respectively. However, the isolated L3 loop is not sufficient to fully support this interaction. The SMAD-receptor interaction may require cooperativity provided by the oligomeric state of both the receptors and the SMADs.

### *Activated SMAD Complexes*

Receptor-phosphorylated SMADs associate with Smad4, which functions as a shared partner required for transcriptional activation (Figure 6). Smad1 associates with Smad4 in response to BMPR-I activation (68, 201) and with Smads 2 and 3 in response to T $\beta$ R-I or ActR-IB activation (201, 204). Smad4 can associate with these SMADs in yeast, which suggests that the interaction is direct (206). Based on structural considerations and the observation that mutations in the Smad4 L3 loop abolish the ability of Smad4 to associate with Smad2, the Smad4 L3 loop appears to mediate the association with receptor-activated SMADs (214). SMAD L3 loops, therefore, are implicated in two distinct types of interactions: (a) interaction with the receptors in the case of receptor-regulated SMADs and (b) interaction with receptor-activated SMADs in the case of Smad4. Functional interactions between receptor-regulated SMADs and a Smad4 family member may also occur in *Drosophila* (187) and *C. elegans* (188).

### *Nuclear Localization and Its Regulation*

Nuclear translocation of receptor-activated SMADs occurs with kinetics that closely follow those of the agonist-induced phosphorylation and association with Smad4. Nuclear translocation of Smads 1 and 2 does not require Smad4, as determined using Smad4-defective cells (212). Smad4 is also translocated into the nucleus in response to TGF- $\beta$  or BMP (204, 212), and this translocation requires the presence of Smad1 or Smad2 (212). Thus, it appears that receptor-activated SMADs bind Smad4 in the cytoplasm and carry it into the nucleus (212).

As central mediators of TGF- $\beta$  family signals, SMADs are subject to different types of regulatory mechanisms that integrate and adapt their signaling

potential to the status of the cell. One mode of regulation is by phosphorylation of MAP-kinase sites in the linker region, inhibiting the accumulation of SMADs in the nucleus (216) (Figure 6). Agonists that activate Erk MAP kinases, such as epidermal growth factor (EGF) and hepatocyte growth factor, rapidly induce phosphorylation of Smad1 at serines in four PXSP motifs in the linker region. This phosphorylation is catalyzed by Erk MAP kinases and occurs independently of BMP receptor-mediated phosphorylation of Smad1. Erk-mediated phosphorylation inhibits nuclear accumulation of Smad1 without interfering with the association of Smad1 with Smad4. BMP responses that depend on nuclear accumulation of Smad1 are antagonized by activation of the Erk MAP-kinase pathway (216). This mechanism may underlie the ability of EGF to oppose osteogenic differentiation by BMP2 or the ability of fibroblast growth factor (FGF) to oppose the effect of BMP2 during limb bud outgrowth, digit formation, or tooth development (216). Other receptor-regulated SMADs also have potential MAP-kinase phosphorylation sites in their linker region. SMAD regulation by MAP kinases may therefore be a general phenomenon in the regulation of TGF- $\beta$  signaling.

### *Transcriptional Complexes*

The ability of SMADs to activate transcription was originally detected through the use of GAL4-Smad fusion constructs that activate GAL4 reporter gene (191). GAL4-Smad1 and GAL4-Smad2 constructs activate transcription in response to BMP4 and TGF- $\beta$ , respectively, and their ability to do so requires Smad4, as determined using Smad4-defective cells (212). The first description of a natural SMAD transcriptional complex was made through studies on the activin response factor (ARF), a DNA-binding complex that forms in *Xenopus* embryo explants in response to activin or an endogenous factor, presumably Vg1 (218). ARF binds to a 50-base pair activin-response element (ARE) in the promoter of the homeobox gene *Mix.2*, an immediate-early activin response gene. The first component of ARF to be identified was the DNA-binding protein Fast-1, based on its ability to interact with a hexanucleotide repeat present in the activin-response element (219). Fast-1 is a novel member of the winged-helix family of putative transcription factors (also known as the HNF-3 family or the forkhead family) (220).

Fast-1 associates with Smad2 and Smad4, forming a ternary complex that binds to the ARE (212, 215) (Figure 6). Because Fast-1 is a nuclear protein (219), it probably binds to incoming Smad2-Smad4 complexes in the nucleus. The interaction involves a region within the C-terminal portion of Fast-1 and the MH2 domain of Smad2 (212, 215). Smad4 is not required for the Smad2-FAST1 interaction but contributes two essential functions to the resulting Smad2/Smad4/FAST-1 complex: Through its MH1 domain, Smad4

promotes binding of the complex to DNA, and through its MH2 domain, Smad4 activates transcription (212).

Other members of the winged-helix family might be DNA-binding partners of SMADs. However, members of structurally unrelated families might play this role as well. For example, the *Drosophila* gene *schnurri*, which encodes a zinc-finger protein with homology to various mammalian transcription factors, is genetically implicated in Dpp signaling (221, 222). Another Dpp-activated gene, *Ubx*, is activated via a cyclic AMP response element (CRE) adjacent to a sequence resembling a Mad-binding site (223). Paradoxically, mutation of this Mad-binding site did not interfere with Dpp activation of *Ubx*. SMADs may interact with certain target enhancers without the involvement of DNA-binding subunits (211, 223a,b), but the biological role of these interactions remains to be ascertained.

### Response Elements

Numerous gene responses to TGF- $\beta$  have been described, but only a fraction of these have the characteristics of an immediate transcriptional response. *p15<sup>Ink4b</sup>* and *p21<sup>Cip1</sup>* are cyclin-dependent kinase inhibitors whose rapid introduction in response to TGF- $\beta$  mediates cell cycle arrest (224–227). Clusters of Sp1-like sites near the transcription start site of *p15<sup>Ink4b</sup>* and *p21<sup>Cip1</sup>* score as TGF- $\beta$ -responsive regions in reporter gene assays (228, 229). TGF- $\beta$ -stimulated expression of interstitial collagens and other extracellular matrix proteins underlies important roles of TGF- $\beta$  in development and regenerative processes (11–13). The TGF- $\beta$ -responsive regions of genes encoding such extracellular matrix proteins as collagen  $\alpha 1(I)$  (230), collagen  $\alpha 2(I)$  (231, 232), type 1 plasminogen activator inhibitor (PAI-1) (233, 234), elastin (235), and perlecan (236) resemble Sp1 sites or CTF/NF- $\kappa$ B sites. However, some of these sequences also resemble the Mad-binding element of *vestigial* (211); thus they might be SMAD-binding sites. TGF- $\beta$  and related factors can also cause rapid inhibition of gene transcription. Genes affected in this manner include *c-myc* (14) and the Cdk-activating phosphatase *cdc25A* (237); down-regulation of both genes by TGF- $\beta$  mediates antiproliferative effects. Interestingly, transcriptional activation by TGF- $\beta$  of PAI-1 (238), retinoic acid receptors (239), collagen  $\alpha 2(I)$ , and other genes (238) appears to require AP-1 activity. Furthermore, a Fos-containing repressor has been implicated in the down-regulation of the secretory protease transin/stromelysin by TGF- $\beta$  (240). Whether SMADs participate in all or even a majority of TGF- $\beta$  gene responses is an open question.

### Inhibition by Antagonistic SMADs

Vertebrate Smads 6 and 7 and *Drosophila* Dad are inhibitors of signaling by receptor-regulated SMADs (196, 197, 200, 207–209) (Figure 6). When overexpressed, Smad6 can inhibit BMP signaling and, partially, TGF- $\beta$  signaling

(196), and Smad7 can inhibit TGF- $\beta$  signaling (197, 208) and BMP signaling (197). At lower concentrations, however, Smad6 is a specific inhibitor of BMP signaling in frog embryos and mammalian cells (207). Dad inhibits Dpp signaling in *Drosophila* wing imaginal discs, and when introduced into frog embryos, Dad exhibits anti-BMP effects (209). Inhibitory SMADs participate in negative feedback loops that may regulate the intensity or duration of TGF- $\beta$  responses. Thus, Smad7 expression is rapidly elevated in response to TGF- $\beta$  (197), whereas Dad expression is elevated in response to Dpp (209). The expression of Smads 6 and 7 is elevated by shear stress in vascular endothelial cells (200), a response that might be mediated by autocrine TGF- $\beta$  (241).

Inhibitory SMADs lack a C-terminal SSXS phosphorylation motif, and their N-terminal region has only short segments of MH1 domain homology (196, 197, 207–209). (Smad6 was originally reported as a truncated SMAD structure consisting of the MH2 domain only; see References 200, 242). One mechanism proposed to explain the inhibitory effects of Smads 6 and 7 is based on the observation that each of these SMADs can bind to diverse TGF- $\beta$  family receptors and interfere with phosphorylation of receptor-regulated SMADs (196, 197, 208). This mechanism could account for the nonselective inhibition of BMP effects and TGF- $\beta$  effects observed by overexpression of Smads 6 or 7. It is not known whether physiologic levels of inhibitory SMADs can interfere with receptor binding and phosphorylation of receptor-regulated SMADs.

A different mechanism may underlie the selective inhibition of BMP signaling by Smad6 (207). At low levels, Smad6 does not interfere with receptor-mediated phosphorylation of Smad1 but competes with Smad4 for binding to activated Smad1. In a yeast two-hybrid system, the Smad6 MH2 domain interacts with itself and with the Smad1 MH2 domain, but not with the MH2 domains of Smads 2 or 4. Smad6 binding to receptor-phosphorylated Smad1 yields a transcriptionally inert complex. Therefore, Smad6 appears to act as a Smad4 decoy for BMP-activated SMADs.

#### *Other Kinases in TGF- $\beta$ Signaling*

Components of MAP-kinase cascades mediate numerous responses to mitogens, differentiation factors, inducers of apoptosis, radiation, and osmotic stress (243, 244). Several groups investigating whether TGF- $\beta$  action affects the Erk subfamily of MAP kinases have reported activation (245), inhibition (246, 247), or no change (248) in the activity of these kinases after TGF- $\beta$  treatment. A novel member of this family, TAK1 (TGF- $\beta$ -activated kinase 1) was cloned based on its ability to activate a MAP kinase cascade in yeast (249). In mammalian cells, the activity of a transfected TAK1 is rapidly increased in response to TGF- $\beta$  and BMP4 (249). Overexpression of a kinase-defective TAK1 mutant (249) or a truncated form of the TAK1 activator, TAB1 (250), diminishes the

TGF- $\beta$  response of a reporter gene construct that contains an AP-1 site, implicating TAK1 in these responses. No effect of TAK1 on other TGF- $\beta$  responses has been reported. TGF- $\beta$  activation of the MAP-kinase JNK has been implicated in a similar transcriptional response and tentatively placed downstream of TAK1 (251, 252). However, the JNK-kinase response to TGF- $\beta$  takes several hours, suggesting that JNK is not a primary transducer of TGF- $\beta$  signals in these cells.

### DISRUPTION OF TGF- $\beta$ SIGNALING IN HUMAN DISORDERS

Alterations of TGF- $\beta$  signaling pathways underlie many human disorders. A loss of growth inhibitory responses to TGF- $\beta$  is often observed in cancer cells (253), and a gain of TGF- $\beta$  activity is thought to play a central role in fibrotic disorders characterized by excessive accumulation of interstitial matrix material in the lung, kidney, liver, and other organs (254). Abnormal TGF- $\beta$  activity is also implicated in inflammatory disorders (255–257). The phenotype of mice overexpressing or lacking specific TGF- $\beta$  family members or their receptors has revealed that these alterations have profound effects on the development or homeostasis of many organs (1, 2, 4). However, direct evidence that disruption of TGF- $\beta$  signaling is a cause of human disorders is provided by the following cases, in which genes encoding TGF- $\beta$  family members, their receptors, or SMAD proteins are mutated (Figure 7).

#### *TGF- $\beta$ Receptor Mutations in Cancer*

The effects of TGF- $\beta$  on target cells include several forms of negative regulation of cell proliferation, such as induction of G1 arrest, promotion of terminal differentiation, or activation of cell death mechanisms (14, 258). Numerous reports have described deficiencies in these types of responses in human tumor-derived cell lines (253). Disruption of TGF- $\beta$  signaling could therefore predispose or cause cancer.

This prediction was confirmed by the finding that the TGF- $\beta$  type II receptor is inactivated by mutations in gastrointestinal cancers with microsatellite instability (259, 260). Microsatellite instability is common to many sporadic cancers and results from defects in DNA mismatch repair leading to nucleotide additions or deletions in simple repeated sequences—microsatellites—throughout the genome. The human *T $\beta$ R-II* gene contains one such sequence, a 10-bp polyadenine repeat, starting at nucleotide 709 in the coding region of the extracellular domain. One- or two-base additions or deletions in this repeat occur in most sporadic colon cancers and gastric cancers with microsatellite instability, yielding truncated, inactive *T $\beta$ R-II* products (260–262). Mutations in the *T $\beta$ R-II* polyadenine repeat are also found in colon or gastric tumors from

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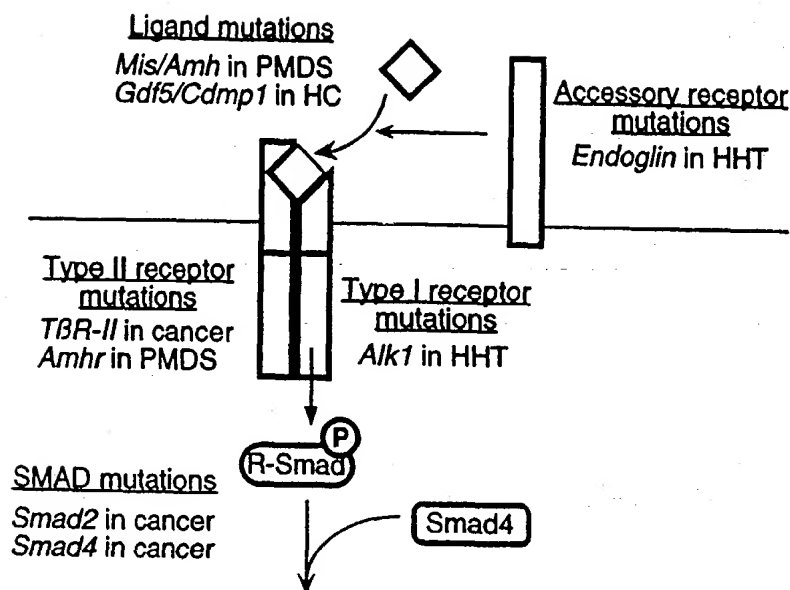


Figure 7 Inactivating mutations in TGF- $\beta$  signal transduction genes in human disorders. *Mis/Amh* or *Amhr* is mutated in persistent Müllerian duct syndrome (PMDS); *Gdf5/Cdmp1* in hereditary chondrodysplasia (HC); *endoglin* or *Alk1* in hereditary hemorrhagic telangiectasia (HHT); *TBR-II* in gastrointestinal cancers with somatic or hereditary microsatellite instability; *Smad2* in colon cancer; and *Smad4* in pancreatic, colon, and other cancers.

individuals with hereditary non-polyposis colon cancer (HNPCC) (263), a familial syndrome characterized by a high incidence of colon, endometrial, and gastric cancers. In most of these cases, both *TBR-II* alleles have mutations in the polyadenine repeat. In some cases, however, the second allele is inactivated by a different mutation, such as (a) an addition of a GT dinucleotide to a GTGTGT sequence in the kinase domain coding region or (b) missense mutations, which are also predicted to inactivate this kinase (260, 261, 263). These results indicate that *TBR-II* shares the two-hit inactivation mechanism of other tumor suppressor genes.

Mutations in the *TBR-II* polyadenine repeat are rare in somatic or hereditary cancers of the endometrium, pancreas, liver, and breast (262, 264, 265) or in myelodysplastic syndrome (266) with microsatellite instability. This provides further evidence that mutations in the *TBR-II* polyadenine repeat are not just a random consequence of microsatellite instability but are specifically selected during the progression of colon and gastric cancers. Mutations elsewhere in

*TβR-II* have been described in T-cell lymphoma, gastric cancers, and head and neck carcinomas (267–269).

### *SMAD Mutations in Cancer*

The TGF- $\beta$  signaling network is also disrupted in cancer by mutations in *Smad2* and *Smad4/DPC4*. *Smad4/DPC4* was originally identified as a candidate tumor suppressor gene in chromosome 18q21 that was somatically deleted or mutated in half of all human pancreatic carcinomas (189). Biallelic *Smad4/DPC4* inactivation also occurs in a significant proportion of colorectal tumors (270, 271). *Smad4/DPC4* is infrequently mutated in breast (272), ovarian (272), head and neck (273), prostatic (271), esophageal, and gastric cancers (274). In the mouse, *Smad4* inactivation causes intestinal tumors in concert with inactivation of another tumor suppressor gene, *APC* (274a). *Smad2* is also located at 18q21, and it too is the target of inactivating mutations in colon cancer (193, 275, 276). Loss of TGF- $\beta$  responsiveness in colon cancer therefore may be due to mutations in *TβR-II*, *Smad2*, or *Smad4/DPC4*. Interestingly, the preponderance of *Smad4/DPC4* mutations in pancreatic cancer, together with the low frequency of mutations in *TβR-II* in these tumors (264), raises the possibility that loss of *Smad4* function may be selecting for resistance to an endogenous factor other than TGF- $\beta$  itself.

*Smad2* and *Smad4/DPC4* are inactivated in cancer by missense mutations, nonsense mutations, small deletions, frameshift mutations, or loss of the entire chromosomal region. Most of the missense mutations described fall in the MH2 domain (214), a region that is also the target of mutations in *Mad*, *sma-2*, and *sma-3* inactive alleles (186, 188). The location of these mutations is consistent with the effector role of the MH2 domain in SMAD signaling. Resolution of the crystal structure of the *Smad4* MH2 domain has revealed that tumor-derived missense mutations in this domain often affect amino acids that are critical for monomer-monomer interactions within the *Smad* trimer (214). Such mutations weaken *Smad* homo-oligomerization and prevent TGF- $\beta$ -induced *Smad2*-*Smad4* association. Less frequently, tumor-derived mutations destabilize the folding of the MH2 domain (214). Tumor-derived missense mutations have also been identified in the MH1 domains of *Smad2* and *Smad4/DPC4*. These mutations inactivate SMAD function by increasing the affinity of the MH1 domain for the homologous MH2 domain, locking the molecule in an inhibited conformation (206). Several mutations from inactive alleles of *Mad* or *sma* genes map to the region corresponding to the L3 loop and are predicted to interfere with heteromeric *Smad* interactions (214) or *Smad*-receptor interactions (217). However, no such mutation has been described in human SMAD genes.



*GDF5/CDMP1 Mutations in Hereditary Chondrodysplasia*

The phenotypes of mice defective in specific members of the BMP and GDF subfamilies have indicated that despite their similar activities in tissue culture, each of these factors is rate limiting for a distinct subset of developmental processes, including the development of specific skeletal components (1, 2, 4). One example of this is provided by the finding that the *brachypodism* phenotype in mice is due to inactivating mutations in the *Gdf5* gene (277, 278). *Brachypodism* mice have numerous alterations in the length and number of bones in the limbs but retain a normal axial skeleton (277). This finding raised the possibility that the human GDF5 homologue, known as cartilage-derived morphogenetic protein 1 (CDMP1), might likewise be involved in skeletal abnormalities. This possibility was confirmed with the identification of a frameshift mutation in *Cdmp1* in individuals with the recessive chondrodysplasia syndrome, Hunter-Thompson type acromesomelic chondrodysplasia (279). The mutation found in this study is a 22-bp insertion in the mature region of CDMP1 and most likely yields an inactive product. The abnormalities in affected individuals are restricted to the limbs and are most severe in the distal bones, which are short and dislocated (279).

*ALK1 and Endoglin Mutations in Hereditary Hemorrhagic Telangiectasia*

The accessory receptor endoglin (103) and the type I receptor ALK1 (33) are highly expressed in vascular endothelial cells. The genes encoding these products have been identified as the targets of inactivating mutations in human hereditary hemorrhagic telangiectasia (124–126). This disorder is characterized by epithelial vascular dysplasia and a high propensity to hemorrhage in the nasal and gastrointestinal mucosa (280). The autosomal dominant nature of this disorder argues that maintenance of appropriate endoglin and ALK1 levels is crucial for vascular homeostasis. The similarity of the phenotypes caused by mutations in either gene suggests that both receptors function in a common pathway controlling the development of the vascular wall. Because endoglin and ALK1 are not effective at binding TGF- $\beta$  (33, 34, 110), it is possible that these two receptors mediate the action of an as yet unidentified TGF- $\beta$  family member in the vasculature.

*MIS and MIS Receptor Mutations in Persistent Müllerian Duct Syndrome*

During the development of the reproductive tract in mammals, the Müllerian duct gives rise to the uterus, fallopian tubes, and upper vagina (15, 16). Regression of the Müllerian duct in males is mediated by MIS/AMH from the

Sertoli cells of the fetal testis acting via its receptor, AMHR, on the mesenchymal cells adjacent to the Müllerian duct epithelium (23, 43, 281). Disruption of this process leads to the appearance of internal pseudohermaphroditism with uterine and oviductal tissues in affected males, a disorder known as persistence of Müllerian duct syndrome (PMDS) (282). PMDS has been shown to result from inactivating mutations in either *Mis/Amh* (283–285) or *Amhr* (285–287). A 27-bp deletion in *Amhr* is a common cause of PMDS (288). The phenotypes of mutations in *Mis/Amh* and *Amhr* are essentially the same, and they are copied in mice defective in the ligand, the receptor, or both (289). These observations suggest that unlike other TGF- $\beta$  family members, MIS/AMH and its receptor have a highly specific and restricted role during development.

## SUMMARY AND PROSPECTS

Recent progress has led to the elucidation of a general TGF- $\beta$  signaling pathway in which the ligand causes the activation of a heteromeric protein kinase complex that subsequently phosphorylates a subset of SMAD proteins that move into the nucleus, where they activate specific target genes with the agency of DNA-binding partners. The cellular response to a TGF- $\beta$  factor may be determined not only by the receptors and SMAD isoforms present in the cell but also by the available repertoire of DNA-binding partners. The response is further modulated by regulators of ligand binding, receptor activity, SMAD activation, or nuclear localization. All the central components of these pathways and many of their regulators are novel proteins of previously unknown function.

The combinatorial interactions that configure such TGF- $\beta$  signaling pathways provide a basis for understanding the multifunctional nature of these factors. In principle, now it should be possible to determine which combination of receptors, SMAD proteins, and DNA-binding partners leads to each particular TGF- $\beta$  gene response. This signaling process is based on a succession of discrete protein-protein and protein-DNA interactions. The structural elements that mediate each contact can now be investigated to ascertain how signaling specificity is enforced in the pathway. These protein interactions are of limited strength; thus they seem good candidates as drug targets. This prospect is interesting, for either gain or loss of TGF- $\beta$  signaling processes underlies various developmental disorders, several forms of cancer, and other ailments in humans.

The progress made allows us to explain, in general terms, how a TGF- $\beta$  signaling pathway works. However, what is described here will likely become, with time, only part of the explanation as the complexity of this pathway is exposed in full. We might yet learn that type II receptors phosphorylate a different set of transducers, or that type I receptors have other substrates besides SMAD proteins, or that SMADs have other functions besides activating

transcription. Furthermore, the recent emphasis on the transcriptional effects of TGFs and family members may have sidestepped other important responses to these factors; it is time to investigate these other responses as well. Clearly then, more work and more surprises lie ahead. However, the recent elucidation of the first contiguous TGF- $\beta$  signaling pathway is a major milestone in this field and provides the framework for future research.

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